ISSN 1330-9862
(FTB-2753)

original scientific paper

Optimization of the Production of Polygalacturonase from *Aspergillus kawachii* Cloned in *Saccharomyces cerevisiae* in Batch and Fed-Batch Cultures

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Received: September 15, 2010
Accepted: February 24, 2011

Summary

Polygalacturonases (PG; EC 3.2.1.15) catalyze the hydrolysis of pectin and/or pectic acid and are useful for industrial applications such as juice clarification and pectin extraction. Growth and heterologous expression of recombinant *Saccharomyces cerevisiae* which expresses an acidic PG from *Aspergillus kawachii* has been studied in batch and fed-batch cultures. Kinetics and stoichiometric parameters of the recombinant yeast were determined in batch cultures in a synthetic medium. In these cultures, the total biomass concentration, protein concentration, and enzyme activity achieved were 2.2 g/L, 10 mg/L, and 3 U/mL, respectively, to give a productivity of 0.06 U/(mL·h). In fed-batch cultures, various strategies for galactose feeding were used: (*i*) after a glucose growth phase, the addition of a single pulse of galactose which gave a productivity of 0.19 U/(mL·h); (*ii*) after a glucose growth phase, a double pulse of galactose at the same final concentration was added, resulting in a productivity of 0.21 U/(mL·h); (*iii*) a simultaneous feeding of glucose and galactose, yielding a productivity of 1.32 U/(mL·h). Based on these results, the simultaneous feeding of glucose and galactose was by far the most suitable strategy for the production of this enzyme. Moreover, some biochemical characteristics of the recombinant enzyme such as a molecular mass of ~60 kDa, an isoelectric point of 3.7 and its ability to hydrolyze polygalacturonic acid at pH=2.5 were determined.

Key words: *Aspergillus kawachii*, acid polygalacturonase, heterologous expression, fed-batch culture, co-substrate feeding

Introduction

Pectins are important components of plant cell walls, and facilitate many of the functions performed in plant tissues. The main structural feature of the pectin molecule is a linear chain of β-(1-4)-linked D-galactosyluronic acid residues in which various proportions of acid groups are present as methyl esters. Pectins are used in the food and pharmaceutical industries as functional ingredients because of their ability to form gels at low concentrations and increase the viscosity of liquid foods (*1*). Pectinolytic enzymes are widely used in the food and beverage industries to clarify fruit juices and wines, to improve cloud stability in fruit and vegetable nectars, and to increase the protein content in fruit juices. The ability to degrade pectins could be used as an alternative method of pectin extraction from by-products of the fruit industry and also to macerate vegetable tissues in order to produce single-cell suspensions that can be used to pre-

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pare fruit nectars, vegetable purees and baby and geriatric foods (*2*).

Some strains of *Aspergillus* genus are known to be capable of growing in extremely acidic environments (pH=2) and to produce acid-stable enzymes that are characteristic of this genus and have adequate properties for a potential use on an industrial scale. *Aspergillus kawachii* IFO 4308 produces several acidic depolymerases such as α-amylases and glucoamylases (*3*), xylanases (*4*), and acidophilic proteinases (*5*). An acidophilic polygalacturonase (PGase) also expressed by *A. kawachii* has been reported by Contreras Esquivel *et al. (6*) and was referred to by the authors as PG1.

A. kawachii, like many other fungi, produces small quantities of PG1. Even when culture conditions were optimized for enzyme expression, the obtained activity was not sufficient for industrial purposes. For this reason, the cloning of the *pg1* gene and the appropriate genetic engineering for its overexpression have been developed in order to increase enzyme production. *Saccharomyces cerevisiae* has certain properties that have been shown to enable the expression of heterologous proteins of biotechnological interest. Gene expression systems in yeast offer a broader range of potential industrial applications than the corresponding bacterial systems do. For example, yeasts are as easy to manipulate and grow as bacteria but, unlike the latter, their subcellular organization is capable of the posttranslational processing of many heterologous proteins (*7*). Furthermore, *S. cerevisiae* has been universally recognized as having the GRAS status (Generally Recognized As Safe) and moreover, secretes a small number of native proteins into the culture medium at relatively low levels. This latter property would facilitate the purification of any recombinant protein.

When an expression system containing the galactose-inducible promoter is used, cells are usually first cultivated with glucose as the carbon and energy source (CES) to obtain the necessary biomass before the previously cloned gene is induced by the addition of galactose. The molar ratio of galactose to glucose and the absolute concentration of galactose are the key parameters in determining the level of gene expression (*8*,*9*).

In this paper, the heterologous expression in *S. cerevisiae* of an acid PGase from *A. kawachii*, in batch and fed-batch cultures, and some biochemical characterization of this recombinant enzyme are reported. To the authors' knowledge, this is the first report on the expression of a pectinase gene from *A. kawachii* in *S. cerevisiae.*

Materials and Methods

Chemicals

Polygalacturonic acid (PGA) was obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were obtained from Anedra (Buenos Aires, Argentina) and were of analytical grade. All of these products were used as recommended by the manufacturers. Citric phosphate buffer (CPB) used in this study contained 50 mM citric acid and 25 mM Na_2HPO_3, pH=2.5.

Strains, plasmids, and culture conditions

Saccharomyces cerevisiae INVSc1, genotype Sc1: MATa *his3Δ1 leu2 trp1-289 ura3-52* MATα *his3Δ1 leu2 trp1-289 ura3-52* (Invitrogen, Buenos Aires, Argentina) carrying the plasmid pYES2:*pg1ΔI*, called INVSc1:*pg1ΔI* was used for the expression of the cloned *pg1* gene.

S. cerevisiae INVSc1 was cultured in synthetic complete medium without uracil (SC-URA) according to the manufacturer's protocol. The medium contained (in g/L): CES 10.0, urea 1.25, KH_2PO_4 1.0, $MgSO_4$ 0.5, NaCl 0.1, $CaCl_2$ 0.1 and vitamin stock solution 1.0 mL/L, amino acid stock solution 10.0 mL/L and trace element stock solution 1.0 mL/L.

Expression studies

Batch cultures in flasks

The expression of *pg1* in submerged cultures in flasks was carried out using two different protocols. Preliminary studies were conducted according to the Invitrogen recommendations (raffinose preparation protocol). The transformed *S. cerevisiae* was cultured overnight in SC--URA medium with raffinose as the CES. An aliquot of the culture was centrifuged at 1500×*g* for 15 min and the pellet was resuspended and inoculated into 100 mL of induction medium (SC-URA medium with 10.0 g/L of raffinose as the CES, containing 20.0 g/L of galactose as the inducer) at an initial $A_{600\ nm}$ of 0.4. Samples (5 mL) were thereafter taken every 2 h for a total of 10 h. The $A_{600\ nm}$ and pH were measured and an aliquot was centrifuged and frozen for further analytical assays and determination of enzyme activity.

A second strategy (the glucose protocol) was also used. Transformed *S. cerevisiae* cells were cultured overnight in SC-URA medium with glucose as the CES. An aliquot of the culture was centrifuged at 1500×*g* for 15 min. The pellet was resuspended and inoculated into 100 mL of SC-URA medium plus 10.0 g/L of glucose as the CES. When glucose was depleted and exponential growth ceased, the culture was subjected to starvation for 2 h to avoid glucose repression, and the expression system was then induced by the addition of galactose to a final concentration of 20.0 g/L. The sample processing was similar to that of the raffinose protocol.

Bioreactor cultures

Batch and fed-batch cultures were carried out in a 1.5-litre BioFlo 310 Benchtop Bioreactor (New Brunswick Scientific, Edison, NJ, USA) with SC-URA medium following the glucose protocol at 30 °C, with aeration (1.0 vvm) and agitation (450 rpm). The culture pH was measured with a glass electrode (Mettler Toledo AG, Greifensee, Switzerland) and automatically controlled at either 5.5 (growth phase) or 4.5 (induction phase) with 0.5 M H_2SO_4 or 1 M NaOH. The dissolved oxygen was measured with a polarographic-type electrode (Mettler Toledo). The outlet gas was analyzed with a paramagnetic O_2 detector (Series 1100, Servomex, Norwoods, MA, USA) and an infrared CO_2 detector (Pir 2000, Horiba, Japan). The O_2 uptake and CO_2 production rates were calculated according to Cooney *et al.* (*10*).

In order to obtain the culture to be seeded for fermentation, cells from the glycerol stock kept at –70 °C were first inoculated into SC-URA solid medium and grown overnight, then inoculated into 100 mL of liquid SC-URA medium, grown once again overnight, and then

collected by centrifugation. After resuspension, these cells were inoculated into the fermentor containing SC-URA medium.

Batch fermentation was carried out in 1.1 L of SC--URA medium. Two hours after glucose depletion, an induction similar to that of the glucose protocol was carried out. The kinetics and stoichiometric parameters of *S. cerevisiae* INVSc1:*pg1ΔI* growth were determined during the growth phase (with glucose as the CES).

Fed-batch fermentations were carried out using three different feeding and induction strategies: (*i*) after a glucose growth phase of 30 h at a feeding rate of 5.2 mL/h and a concentration of 300 g/L of glucose, the culture was induced with a single pulse of galactose at a final concentration of 10 g/L; (*ii*) after a similar glucose growth phase, the culture was induced with two sequential (separated by 5 h) pulses of galactose (10 g/L); and (*iii*) a simultaneous feeding of both glucose and galactose (100 g/L) as the CES and inducer, respectively. The same conditions of batch fermentation were maintained during feeding phase except that the shaking speed was regulated according to the dissolved O_2 concentration.

The fed-batch protocol (with respect to the glucose concentration and the feeding rate) was designed according to the equations derived from the mass balances for the substrate and biomass in carbon-limited cultures by means of the kinetic and stoichiometric parameters calculated in the batch cultures.

Enzyme assay

PG1 activity was determined as described by Contreras Esquivel and Voget (*11*) with minor modifications. The enzyme activity was measured at pH=2.5 with 2.0 g/L of PGA in CPB as the substrate. When low activity was expected, the reaction time was extended to up to 2 h (*12*). One unit of enzyme activity was defined as the production of 1 μmol of reduced sugar as galacturonic acid per min.

Enzyme characterization

The influence of pH on the enzyme activity was determined with 2.0 g/L of PGA in CPB as the substrate after varying the pH from 2.0 to 5.0. The thermal stability of the expressed PG1 was evaluated by incubating the enzyme at pH=2.5 in CPB for 10 h at different temperatures. Samples were taken every 2 h and kept at 0 °C until the time of assay.

The protein concentration was measured by the Bradford technique (*13*) with Coomassie Brilliant Blue G-250 as the chromophore and bovine serum albumin as the standard. SDS-PAGE was carried out in a Mighty Small II Unit (Hoefer SE 260 mini-vertical gel electrophoresis unit, Amersham Pharmacia Biotech, Amersham, Buckinghamshire, UK) (*14*), while isoelectric focussing was performed with an LKB 2117 Multiphor II electrophoresis unit (Amersham Pharmacia Biotech) on a 7.5 % (by mass per volume) polyacrylamide gel containing 5 % (by volume) Pharmalyte™ (Amersham Pharmacia Biotech) (pH=2.5–5.0) according to the instructions of the manufacturer. PG1 was visualized after staining with Coomassie Blue.

Results and Discussion

Expression of pg1ΔI gene in yeast

Batch cultures in flasks

In order to study the ability of INVSc1:*pg1ΔI* to produce the heterologous PG1, two series of galactose induction experiments were carried out in Erlenmeyer flasks. In the first experiment, the protocol suggested by Invitrogen was used without any modification. Both clones were cultured aerobically at 30 °C in 100 mL of SC-URA medium with raffinose as the CES up to the CES-depletion point. Raffinose was used as the CES because this trisaccharide does not repress the GAL10 promoter as glucose or other sugars do. The cultures were then supplemented again with raffinose and induced with galactose. A PGase activity of 2 U/mL was obtained after 24 h of induction under these conditions (Fig. 1). Although

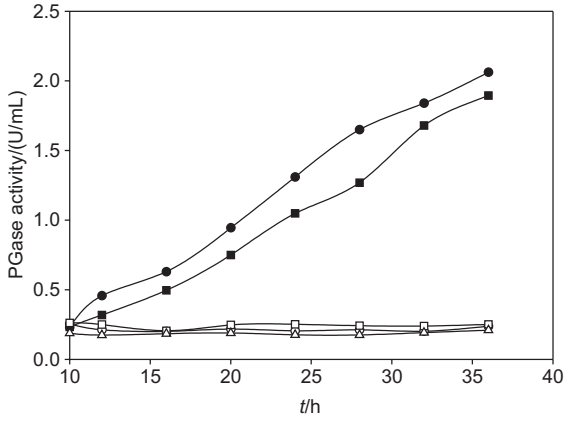

Fig. 1. PGase expression by recombinant *S. cerevisiae* following the raffinose protocol. Batch cultures in SC-URA medium were induced with galactose after 10 h of exponential growth in raffinose. ● clone 1 (induced), ■ clone 2 (induced), ○ clone 1 (noninduced), □ clone 2 (noninduced), △ negative control (without the *pg1* gene)

this value may seem low, the activity is 100 times higher than that of the PGase obtained in *A. kawachii* cultures (*6*). A culture of INVSc1 transformed with pYES2, not containing the *pg1* gene, and incubated under similar conditions as a negative control exhibited no induction.

Since raffinose is too expensive to be used as the CES in an industrial fermentation process, a second strategy for induction was carried out. INVSc1:*pg1ΔI* was first grown in SC-URA medium with glucose as the CES, then subjected to 2 h of starvation before a final galactose induction in order to avoid glucose repression. Since no major differences in PGase induction between the two experimental approaches were observed (Fig. 2), it was decided to use the second protocol for all further studies. Because of the potential industrial application of this enzyme, a low-cost medium would be critical, given the degree of scaling-up necessary for the industrial production process.

Batch cultures in a bioreactor

Fig. 3a shows the time course of biomass growth and substrate consumption in a batch culture. From these data, a maximum specific growth rate (μ_{max}) of 0.28 h^{-1} was

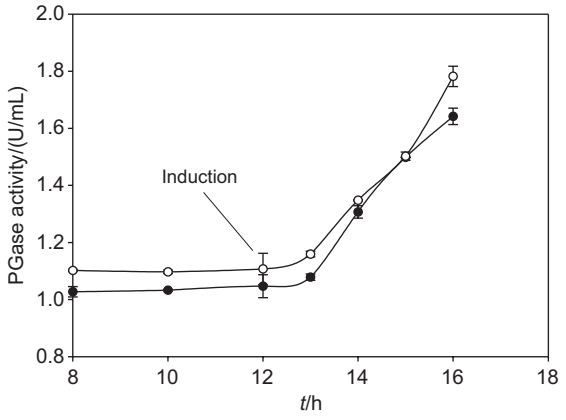

Fig. 2. Induction of *pg1* gene cloned in *S. cerevisiae* grown in raffinose or glucose as CES. Induction was carried out after 8 h of exponential growth. ● glucose, ○ raffinose

calculated, which is lower than that reported for wild-type *Saccharomyces cerevisiae* in synthetic medium (*15*). The decline in the μ_{max} value is understandable in view of the metabolic burden caused by the foreign construct. The dissolved oxygen concentration was always above 60 % saturation, thus insuring oxygen levels were non-limiting. Fig. 3b shows the time course for the rates of

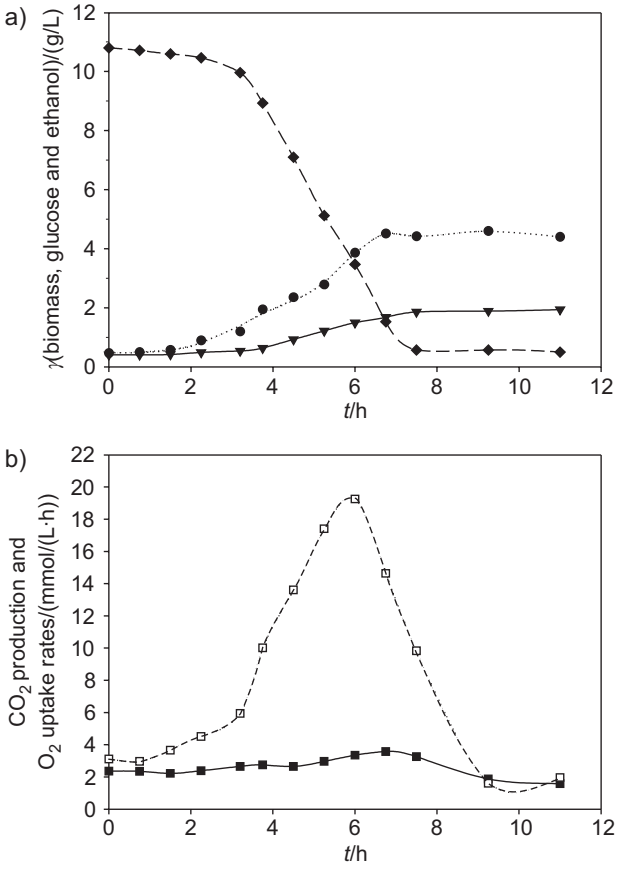

Fig. 3. Profiles of the rates of growth, glucose consumption, ethanol production, volumetric O_2 consumption, and CO_2 production during the batch cultivation of *S. cerevisiae* INVSc1 containing the pYES2:*pg1ΔI* construction: a) ◆ remaining glucose, ▼ biomass concentration, ● ethanol concentration; b) □ CO_2 production rate, ■ O_2 consumption rate

O_2 consumption and CO_2 production. The respiratory quotient was always above 1, typical of an overflow metabolism (*i.e.* from the Crabtree effect) (*16*).

Table 1 shows the stoichiometric parameters of the culture. The carbon and energy balances were calculated according to Erickson *et al.* (*17*) for product-generating cultures and found to be close to unity, indicating that only biomass, ethanol, and CO_2 are produced during cultivation under these conditions.

Once the growth phase had ended, PGase expression was induced by a pulse of galactose as described above (for the glucose protocol). After the induction phase, the total biomass concentration, protein concentration, and enzyme activity were 2.2 g/L, 10 mg/L, and 3 U/mL, respectively, to give a productivity of 0.06 U/(mL·h). This enzyme concentration is higher than that obtained with batch cultures in flasks, probably as a result of the degree of environmental control (*e.g.* pH, dissolved O_2 levels) attained in the bioreactor. Contreras Esquivel and Voget (*11*) reported that PG1 is stable only within a pH range between 2.5 and 5.0. For this reason, the pH control was extremely critical in maintaining the enzyme stability during the expression phase. Furthermore, the biomass yield during this last phase was lower than that obtained during the growth phase. Considering that glucose and galactose are similar as CESs, the decrease in $Y_{X/S}$ (Table 1) could result from the metabolic burden caused by the expression of the foreign gene.

Table 1. Stoichiometric parameters of *S. cerevisiae* batch culture. The carbon and energy balances were calculated according to Erickson *et al.* (*17*) for product-generating cultures

$\frac{Y_{X/S}}{\text{mol}}$	$\frac{Y_{CO_2/S}}{\text{mol}}$	$\frac{b}{\text{mol}}$	$\frac{Y_{P/S}}{\text{mol}}$	$\frac{a}{\text{mol}}$	CB	γ_B
0.173	0.291	0.082	0.523	0.0345	0.95	1.05

$Y_{X/S}$=biomass yield, $Y_{CO_2/S}$=carbon dioxide yield, $Y_{P/S}$=ethanol yield, *a*=urea coefficient, *b*=oxygen coefficient, CB=carbon balance, γ_B=reduction degree (energy balance)

Fed-batch cultures in bioreactor

The optimal cultivation method should lead to high volumetric productivity, high final product concentration, stability and reproducibility of the process. If it is possible, low-cost substrates should be used (*18*). Most protein production processes are based on fed-batch protocols. In an attempt to increase PG1 productivity, a number of fed-batch fermentations using different strategies for introducing galactose into the growing culture were conducted. In order to control the rate of glucose feeding in fed-batch cultures so as to avoid an accumulation of the CES and a consequent production of ethanol through the Crabtree effect, fundamental fermentation parameters were estimated from the batch-culture data (Table 1). The desired final biomass concentration was 15 g/L. For this level of biomass, the corresponding parameters were: X_0=2.20 g/L, V_0=0.8 L and V_f=1.12 L, S_R=320 g/L, F=10 mL/h, μ_0=0.28 h^{-1}, $Y_{X/S}$=0.173 mol/mol, where X_0, V_0 and μ_0 are biomass concentration, volume and specific

growth rate at initial condition respectively, V_f is final volume, S_R is substrate feeding concentration, F is feeding flux and $Y_{X/S}$ is biomass yield.

The various strategies used for galactose feeding were described in Materials and Methods. In the first two strategies, the final biomass concentration obtained in this culture system before induction was 11.11 g/L. Once the glucose feeding was halted, the expression of the enzyme was induced by the addition of galactose. With the strategy described in (*i*), after the glucose depletion from the medium and the addition of a single pulse of galactose, the final PG1 concentration and total protein content obtained were 12.2 U/mL and 67 mg/L, respectively, after 63 h of culture to give a productivity of 0.19 U/(mL·h). These yields are higher than those obtained in batch cultures. The reason for the improved PG1 productivity was probably the greater biomass concentration achieved in this culture system.

Strategy described in (*ii*), with the addition of a double pulse of galactose, gave a final PG1 concentration and a total protein content of 16 U/mL and 73.6 mg/L, respectively, after 78 h of culture, corresponding to a productivity of 0.21 U/(mL·h). This strategy therefore resulted in only a marginal increase in PG1 productivity relative to strategy (*i*).

Strategy (*iii*), involving simultaneous growth and induction phases, was based on the idea that during the growth phase, glucose is used as the CES and its concentration thus becomes almost negligible; so that the galactose promoter should not be repressed, and the *pg1* gene can therefore be expressed (*19*). This feeding strategy yielded a final PG1 concentration and a total protein content of 50.0 U/mL and 54 mg/L, respectively, after 38 h of culture to give a productivity of 1.32 U/(mL·h).

When comparing the results of these three approaches to the level of PG1 production by the fed-batch mode, it was concluded that the simultaneous feeding of glucose and galactose was by far the most suitable strategy for the generation of this enzyme instead of a step-wise feeding of galactose, as suggested in previous reports (*20*).

An industrial production process must be capable of yielding large amounts of products while still keeping the fermentation time as short as possible; moreover, the substrate should be completely utilized at the same time since the cost of raw material is the predominant manufacturing expense. In addition, an industrial production strategy must take into account the regulation of expression of the specific gene in question, a feature determined by its promoter. For these reasons, this last approach would appear to be the most promising one for the recombinant production of PG1 in large-scale experiments.

Partial characterization of the recombinant protein

Some biochemical properties were determined. The recombinant protein had a molecular mass of 60.1 kDa (Fig. 4) and an isoelectric point of 3.7.

With PGA as substrate, maximum activity occurred at around pH=4.0, but the enzyme activity was still recorded at pH=2.0 (at 20 % of the maximum activity), where the PGA substrate precipitates out of the solution (Fig. 5a). Moreover, the recombinant PG1 retained no activity at pH=5.0, similar to the previously reported pH

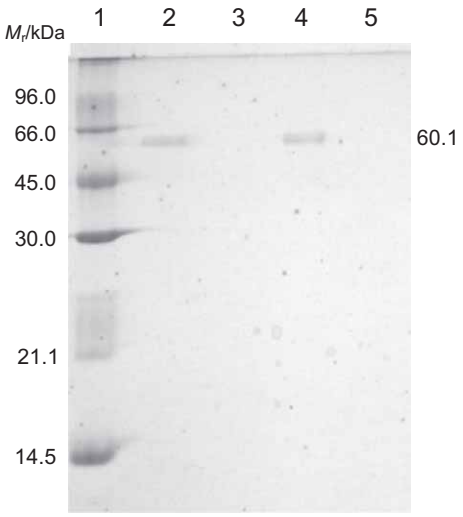

Fig. 4. SDS-PAGE of the recombinant PG1. Lane 1: low-molecular-mass protein standard (GE), lane 2: recombinant PG1 from clone 1, lane 3: negative control (noninduced clone 1), lane 4: recombinant PG1 from clone 2, lane 5: negative control (noninduced clone 2)

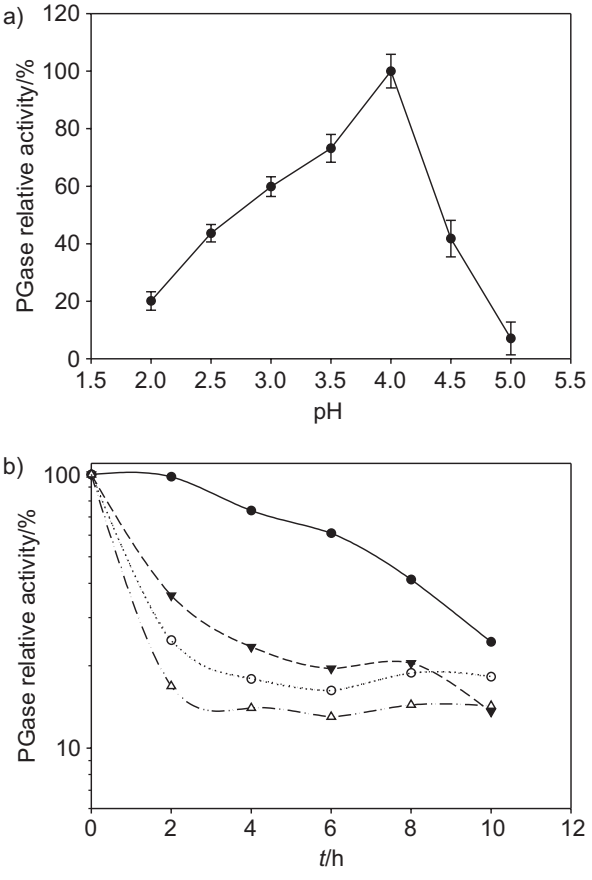

Fig. 5. Biochemical characterization of the recombinant PG1: a) effect of pH on the activity of *S. cerevisiae* PG1. CPB buffer adjusted to pH=2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 was used in the measurement of enzymatic activity under the standard conditions; b) effect of temperature on PG1 stability. The enzyme solution was incubated at the indicated temperatures, and at the selected time intervals the samples were taken and chilled in an ice bath. The residual PG1 activity was then measured under standard conditions. ● 50, ▼ 55, ○ 60, and △ 70 °C

profile for the nonrecombinant enzyme (*11*). The activity of PG1 at pH=2.5 would render its function for the maceration of vegetable tissues and their subsequent pectin extraction since that low pH would minimize any possible microbial contamination within the preparation.

The thermal stability of PG1 was studied at pH=2.5. PG1 activity proved to be highly stable at 50 °C, with only 15 % of the activity lost after 10-hour incubation at that temperature, even when no protective agent was added (Fig. 5b). Temperatures higher than 55 °C, however, resulted in a rapid loss of catalytic activity. Finally, the enzyme in CBP buffer, pH=2.5, without any protective agent, was not inactivated after two cycles of freezing and thawing.

Conclusions

Since PG1 and PgaA showed similar properties, and particularly because the PGase expressed by *S. cerevisiae* has activity at pH=2.5 but not at pH=5.0, we assumed that the two enzymes represent one and the same protein (*6*,*11*). An optimization of the conditions for recombinant PG1 production in continuous cultures is currently under investigation in order to evaluate the prospect of employing this enzyme in biotechnological processes.

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